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Anti-inflammatory activity of the basolateral fraction of Caco-2 cells exposed to a rosemary supercritical extract

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## Highlights

- Uptake of carnosic acid and carnosol from rosemary supercritical extract.
- Basolateral fraction of the extract showed an important anti-inflammatory activity.
- The extract could be used in formulations for prevention of inflammatory diseases.

## Abstract

The anti-inflammatory activity of the basolateral fraction of Caco-2 cells exposed to a rosemary supercritical extract was examined. Uptake of rosemary extract fractions was tested on Caco-2 cell monolayers (2–12 h incubation times) and the quantification of carnosic acid and carnosol was performed by UPLC-MS/MS. Human macrophages were treated with the basolateral fractions and secretion of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 was measured by ELISA. The fractions obtained after 8 and 12 h in absorption experiments caused a significant reduction in excretion of the pro-inflammatory cytokines. This reduction in cytokine secretion levels corresponded to the amounts of carnosic acid and carnosol in the basolateral fractions. Thus, the basolateral fraction of a rosemary supercritical extract showed an important anti-inflammatory activity, providing the basis for increasing the use of supercritical rosemary extracts for the prevention of inflammatory diseases.

## Keywords

Rosemary  
Bioavailable fraction  
Anti-inflammatory activity  
Supercritical extraction

# 1. Introduction

The inflammatory process is a coordinate non-specific response of the immune system induced by a variety of external (microbial) factors or by endogenous products resulting from e.g. cell lysis. Early stages of the counteracting response include the activation of macrophages that secrete signaling molecules, cytokines, as TNF- $\alpha$ , IL-1 $\beta$  or IL-6 with pro-inflammatory activity or IL-10 with anti-inflammatory effect (Barton, 2008; Zhang, 2008). The main function of inflammation is to resolve an infection or repair the damage and restore homeostasis. However, if the regulation of the response fails and develops into a chronic response, the risk of diseases increases such as e.g. atherosclerosis, diabetes, cancer, rheumatoid arthritis or Alzheimer (Akiyama et al., 2000; Medzhitov, 2008; Nathan, 2002). Nowadays anti-inflammatory drugs are on the market that, however, usually exhibit undesirable co-morbidity (Waldner & Neurath, 2009). Food-contained components with anti-inflammatory properties may be attractive in developing nutritional products that can be used in controlling undesirable inflammatory conditions. Consequently, there is an interest for the incorporation of ingredients with beneficial health properties in the food industry that contributes to the treatment or prevention of chronic inflammatory diseases (Sergeant, Piront, Meurice, Toussaint, & Schneider, 2010; Zorrilla et al., 2014).

*Rosmarinus officinalis* L. (rosemary) extracts have been extensively studied since they have been reported to possess antioxidant, antimicrobial, antitumor and anti-inflammatory activities (Ben Jemia et al., 2013; del Pilar Sánchez-Camargo et al., 2014; Dilas et al., 2012; Santoyo et al., 2005; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2008; Yu et al., 2013). These activities have frequently been attributed to the presence of carnosic acid and carnosol, major phenolic diterpenoid components of rosemary leaves (Rau et al., 2006). Kuo et al. (2011) demonstrated that the inhibitory effects of carnosic acid on LPS-induced NO and TNF- $\alpha$  production are related to the suppression of iNOS and COX-2 expression, resulting from inhibition of NF- $\kappa$ B signaling. Carnosol decreases LPS-induced iNOS mRNA and down-regulates the inhibitor NF- $\kappa$ B kinase activity in the mouse macrophage RAW 264.7 cell line (Lo, Liang, Lin-Shiau, Ho, & Lin, 2002). Besides, several authors have reported that rosemary extracts or fractions show a similar or superior anti-inflammatory activity than do carnosic acid or carnosol alone (Yu et al., 2013). To be able to extrapolate results found in vitro to in vivo situation it is important to know in vivo stability, metabolization and bioavailability of rosemary compounds. Doolaege, Raes, De Vos, Verhe, and De Smet (2011) identified carnosic acid in the blood of rats after oral administration. Soler-Rivas et al. (2010), using an in vitro digestion/Caco-2 cell culture model, found no transport across these intestinal cell layer of carnosic acid but only of methyl carnosate. This warrants further research directed to bioavailability of these compounds.

The extraction of rosemary leaves using supercritical CO<sub>2</sub> has been intensively investigated in order to obtain a high percentage of phenolic diterpenes, mainly carnosic acid and carnosol (Carvalho, Moura, Moura, Rosa, & Meireles, 2005; Chang et al., 2008). Several authors have compared supercritical rosemary extracts with the extracts obtained using liquid solvents (ethanol, acetone and hexane) and hydrodistillation. Supercritical extracts presented the highest recovery of carnosic acid compared with the conventional solvent methods (Carvalho et al., 2005). Despite these data, only a few studies have evaluated the anti-inflammatory activity of supercritical rosemary extracts (Kuo et al., 2011; Peng et al., 2007).

The aim of this work was to study the anti-inflammatory activity of the basolateral fraction of Caco-2 cells exposed to a supercritical rosemary extract. In order to simulate the in vivo situation we incorporated the extract to polarized Caco-2 cells grown in a Transwell® plate and subsequently the transported basolateral fraction was exposed to LPS-activated human macrophages (THP-1). Besides, the fate of carnosic acid and carnosol was analyzed. Moreover, in this paper we also studied the anti-inflammatory activity of the

basolateral fraction of a mixture of carnosic acid and carnosol in an attempt to establish a relationship between the anti-inflammatory activity of the extract and these compounds.

## 2. Materials and methods

### 2.1. Chemicals

Carnosol (>95%) was purchased from Sigma-Aldrich (Madrid, Spain) while carnosic acid ( $\geq 97\%$ ) was supplied from Fluka (Madrid, Spain). Ethanol and phosphoric acid (85%) in HPLC grade were purchased from Panreac (Barcelona, Spain) and acetonitrile was obtained from Lab Scan (Dublin, Ireland). CO<sub>2</sub> (N38) was supplied from Carbueros Metálicos (Madrid, Spain). Purified water was obtained from a Milli-Q purification system (Millipore, Madrid, Spain).

### 2.2. Rosemary samples and extraction

Rosemary (*R. officinalis* L.) samples consisted in dried leaves were obtained from an herbalist's shop (Murcia, Spain). Cryogenic grinding of the samples was performed under liquid nitrogen and the ground material was sieved to the appropriate size (between 200 and 600  $\mu\text{m}$ ).

Supercritical rosemary extract was obtained using a pilot-plant supercritical fluid extractor (Thar Technology, Pittsburgh, PA, USA, model SF2000), comprising a 2 L cylinder extraction cell and two different separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. The extraction vessel was packed with 0.5 kg of the cryogenically milled and sieved plant particles. The extraction was performed at 15 MPa and 313 K using 60 g/min CO<sub>2</sub> flow rate and 5% w/w ethanol was employed as cosolvent during 180 min of extraction to obtain one sample from S1 without fractionation.

### 2.3. Chemical characterization of supercritical rosemary extract

HPLC analysis was carried out to determine carnosic acid and carnosol content in the rosemary extract, using a Varian Pro-star equipped with a Microsorb-100 C18 column (Varian, Madrid, Spain). Mobile phase consisted of acetonitrile (solvent A) and 0.1% of phosphoric acid in water (solvent B) applying the following gradient: from 0 min to 8 min, 23% A; increasing from 8 min to 25 min up to 75% A; kept constant during 15 min, and from 40 min to 45 min, initial conditions were gained (23% A). The detection was accomplished by using a diode array detection system (Varian, Madrid, Spain) storing the signal at a wavelength of 230, 280 and 350 nm. Samples were analyzed in duplicate and the obtained average standard deviation was  $\pm 0.13\%$ .

### 2.4. Caco-2 experiments

Caco-2 cells (American Type Culture Collection, ATCC, Barcelona, Spain) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1% nonessential amino acids and 2 mM L-glutamine (Invitrogen, Madrid, Spain) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

The cytotoxic effect of the supercritical rosemary extract and the mixture of carnosic acid and carnosol on Caco-2 cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma), following Mosmann's (1983) method.

For transport experiments, Caco-2 cells were seeded onto twelve-well Transwell® plates (0.4 µm pore size, inserts of 12 mm diameter, Costar, Corning, Madrid, Spain) at a density of  $2 \times 10^4$  cells per insert. The cells were maintained along 21 days, once the monolayer was formed. The integrity of the monolayer was checked by measuring the transepithelial electrical resistance (TEER) (Evon World Precision Instruments, Sarasota, FL, USA). Supercritical rosemary extract or the standards mixture were incorporated in the apical compartment of the Transwell® plate at a concentration of 20 µg/mL (10 µg per well) and incubated for 2, 4, 8 and 12 h at 37 °C. Then, the apical and basolateral samples were collected and stored at -20 °C prior analysis. Cell monolayer was also recovered after the experiments and stored at -20 °C.

## 2.5. Quantification of carnosic acid and carnosol in fractions after absorption experiments

The apical and basolateral samples from Transwell® compartments, after the different incubation times, were freeze dried. The powder obtained was re-suspended in ethanol and centrifuged. Caco-2 cell monolayer samples were centrifuged and the pellet was re-suspended in acetone and sonicated to facilitate disruption of the cells. Finally samples were dried under nitrogen and dissolved in ethanol.

UPLC-MS/MS was employed to detect carnosic acid and carnosol in the apical, basolateral and cell lysates samples, specifically an Accela (Thermo Scientific, San Jose, CA, USA) liquid chromatograph equipped with a DAD and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer via an electrospray interface. The analytical conditions employed consisted of the use of a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 µm) (Thermo Scientific) using as mobile phases acetonitrile (0.1% formic acid, A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 40% B; 6.2 min, 5% B; 6.5 min, 5% B; 7 min, 95% B; 9 min, 95% B (Herrero, Plaza, Cifuentes, & Ibáñez, 2010). The flow rate was 0.4 mL/min while the injection volume was 10 µL. The diode array detector recorded the spectra from 200 to 450 nm. In order to quantify carnosic acid and carnosol, the mass spectrometer was operated in the negative ESI multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM using scan width 0.010 Da and scan time of 0.040 s. Carnosic acid and carnosol transitions were optimized by direct infusion of pure standards. Parent mass of carnosic acid was 331.049 with an ion product mass of 287.175, using a collision energy of 21 and tube lens of 59, while carnosol parent mass was 329.198 with an ion product mass of 285.199, using a collision energy of 17 and tube lens of 51. Carnosic acid and carnosol concentrations in the samples were performed from calibration curves determined by linear regression.

## 2.6. Anti-inflammatory activity of basolateral samples from Caco-2 experiments

Human THP-1 monocytes (American Type Culture Collection, ATCC) were cultured in RPMI 1640 culture medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine (Invitrogen) and 0.05 mM β-mercaptoethanol (Sigma-Aldrich) at 37 °C in 95% humidified air containing 5% CO<sub>2</sub>. Cells were plated at a density of  $5 \times 10^5$  cells/mL in 24 well plates. Differentiation to macrophages (THP-1/M cells) was induced by maintaining the cells in the presence of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h. After differentiation, cells were washed with PBS and incubated with 0.05 µg/mL LPS (Sigma-Aldrich) in presence of the basolateral medium from Caco-2 cells experiments for 24 h. Then, the supernatant was frozen at -80 °C.

The release of IL-1β, IL-10, IL-6 and TNF-α was measured in the supernatants of THP-1/M cells using ELISA kits (BD Biosciences, Madrid, Spain), according to manufacturer's instructions. The color generated was determined by measuring the OD at 450 nm using a multiscanner autoreader (Sunrise Tecan, Barcelona, Spain).

## 2.7. Statistical analysis

All data were expressed as the mean of three determinations  $\pm$  SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's and Bonferroni tests, using Prism program for Windows (Version 5; GraphPad Software, San Diego, CA, USA). P values lower than 0.05 were considered significant.

## 3. Results and discussion

### 3.1. Analysis of supercritical rosemary extract

A supercritical rosemary extract was obtained from a pilot scale plant using 5% of ethanol as a cosolvent, a pressure of 15 MPa and 313 K. These conditions were identical to conditions used in earlier studies by our research group, in order to obtain extracts with a high quantity of carnosic acid and carnosol (Vicente, Martín, García-Risco, Fornari, & Reglero, 2012). The HPLC analysis of the extract showed that the carnosic acid-content of the extract was 256 mg/g, while for carnosol this was 38 mg/g. These results indicated that the extract used in this work contained a higher quantity of carnosic acid than present in supercritical extracts that were used to carry out previous anti-inflammatory studies. Meanwhile carnosol content was similar or slightly higher than that previously reported (Kuo et al., 2011; Peng et al., 2007).

### 3.2. Determination of carnosic acid and carnosol in apical, basolateral and cellular fractions

A supercritical rosemary extract was obtained from a pilot scale plant using 5% of ethanol as a cosolvent, a pressure of 15 MPa and 313 K. These conditions were identical to conditions used in earlier studies by our research group, in order to obtain extracts with a high quantity of carnosic acid and carnosol (Vicente, Martín, García-Risco, Fornari, & Reglero, 2012). The HPLC analysis of the extract showed that the carnosic acid-content of the extract was 256 mg/g, while for carnosol this was 38 mg/g. These results indicated that the extract used in this work contained a higher quantity of carnosic acid than present in supercritical extracts that were used to carry out previous anti-inflammatory studies. Meanwhile carnosol content was similar or slightly higher than that previously reported (Kuo et al., 2011; Peng et al., 2007).

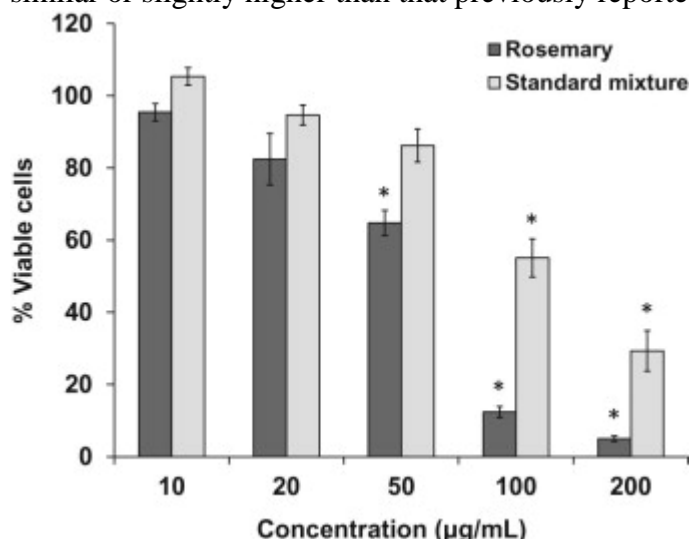


Fig. 1. Viability (%) of Caco-2 cells after 12 h of incubation with rosemary extract or standard mixture of carnosic acid and carnosol. Control cells were grown in media with no treatment. \*Denotes statistical differences between control cells and the other samples at  $p < 0.05$ .

The percentage of carnosic acid and carnosol detected in the exposure experiments is shown in Fig. 2. When rosemary supercritical extract was added to Caco-2 cells, after 2 h the percentage of carnosic acid that was unabsorbed was approximately 75% of the initial amount. The amount detected in the cellular fraction



represented approx. 20% of carnosic acid that was added. An increase in the incubation time (4 h) caused a small reduction in the percentage of carnosic acid that was detectable in the apical compartment until 70%, while in the cellular samples the detectable amount increased to 30%. After 8 h of incubation, carnosic acid was detected also in the basolateral compartment (12% of the added amount), which increased to 25% after 12 h. The increase in the transport of carnosic acid across the Caco-2 cells barrier between 8 and 12 h, seems to be released from the cellular content, as no further decrease was found in the apical concentration. Carnosol was detected inside the cells only after 4 h. After longer incubation (8 h), the carnosol content inside the enterocytes was increased to 8%, and after 12 h up to 20%. Uptake carnosol (3% of the initial concentration added) was only found after 12 h.

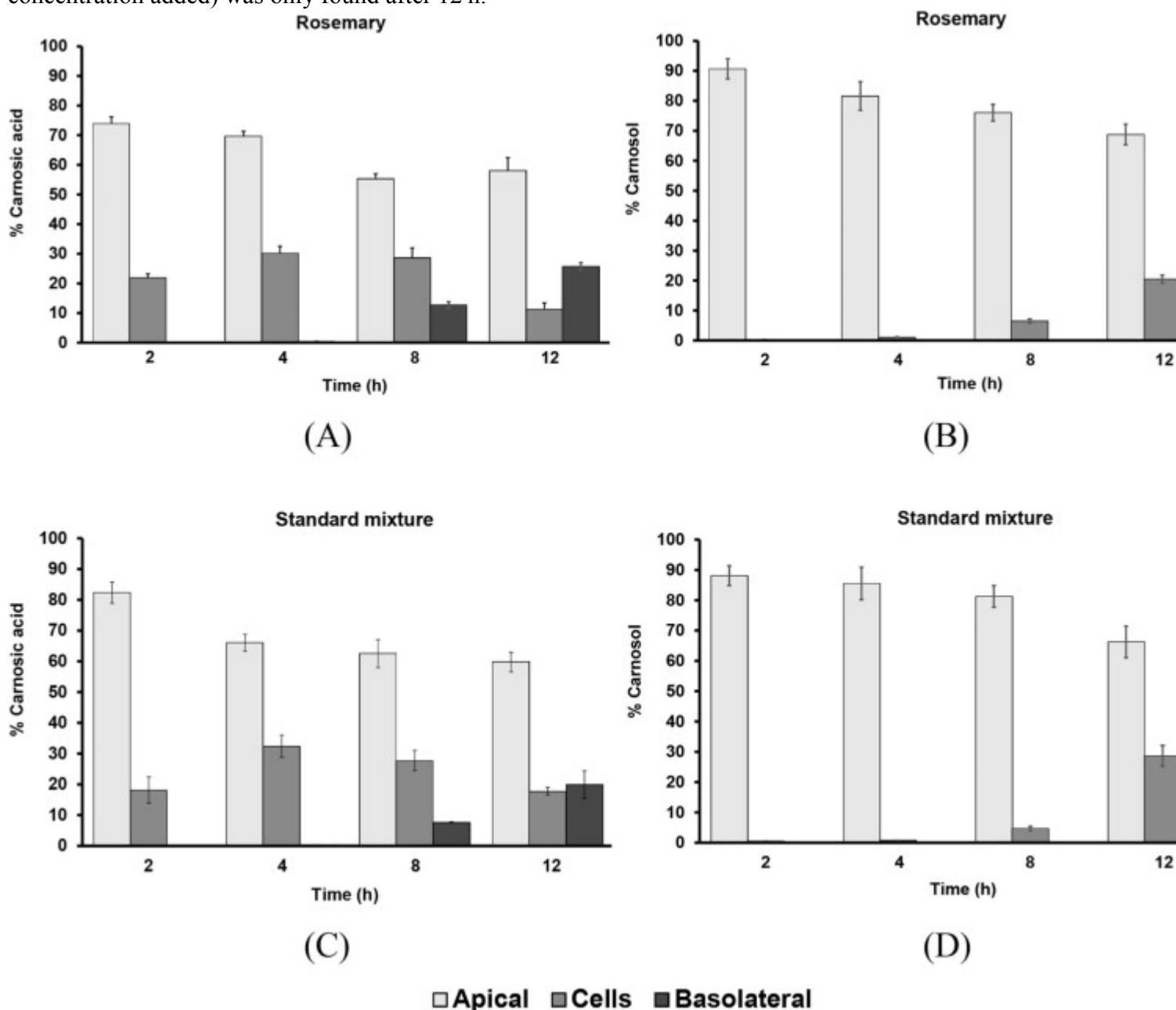


Fig. 2. Carnosic acid (A and C) and carnosol (B and D) percentage recovered from the apical compartment, cells cytoplasm and basolateral compartment from the Transwell® plate after supercritical rosemary extract or standard mixture application at different incubation times.

Fig. 2 also showed the results obtained in the exposure experiments using a mixture of carnosic acid and carnosol at the same concentration in which these compounds were presented in the rosemary extract. The results obtained with this mixture were similar to those obtained from rosemary extract. After 2 h, almost

20% of the initial concentration of carnosic acid could be found in the cytoplasm of the cells. After longer incubation times, the carnosic acid in the cellular fraction was increased (30% after 4 h, 20% after 8 and 12 h). Carnosic acid was detected in the basolateral fraction to a maximum of 20% of the added amount. Finally, carnosol was transported in a low proportion and after 12 h it was found in a low percentage in the basolateral compartment of the Transwell® plates.

These results indicated that carnosic acid and carnosol presented in the rosemary extract showed the same pattern of transport to the mixture of the pure compounds. Furthermore, there was an uptake of carnosic acid to a relevant percentage (20–25%). The bioavailability of this compound has been also reported by Doolaege et al. (2011). These authors indicated that the gastrointestinal absorption of carnosic acid was slow, with a maximum after more than 125 min of treatment and 40% of bioavailability. Similar results were obtained by Yan et al. (2009) after intra-gastric administration of 90 mg/kg of carnosic acid in rats. These authors also reported a slow gastrointestinal absorption and claimed a bioavailability of 65% for carnosic acid in plasma samples. Furthermore, Krause and Ternes (2000) evaluated the carnosic acid content in egg yolk after the supplementation of hen diet with a rosemary extract. However, previous studies in our research group (Soler-Rivas et al., 2010), could not detect carnosic acid and carnosol in the transwell plates basolateral compartment, probably due to the lower sensibility of the analytic method, HPLC-DAD versus UPLC-MS/MS.

### 3.3. Anti-inflammatory activity of the bioavailable fractions

The anti-inflammatory activity of the bioavailable fractions was determined by exposing the basolateral media to THP-1 macrophages. In the present work, this system was used instead of a direct co-culturing Caco-2 and THP-1 since Watanabe, Satsu, Mochizuki, Nakano, and Shimizu (2004) reported that TNF- $\alpha$  produced by macrophages could disrupt the epithelial barrier in a co-culture in transwells of Caco-2 cells and THP-1/M.

During the transport experiments at 2 to 12 h, basolateral compartment of the transwell plates was collected, incubated with LPS-activated macrophages during 24 h and cytokines secretion was measured by ELISA. The results for TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 secretion are presented in Fig. 3. The addition of the basolateral fraction from rosemary extract obtained after 2 and 4 h of Caco-2 uptake experiments, did not induce changes in the cytokine secretion, compared with levels obtained for positive control (macrophages activated with LPS). These results are in agreement with carnosic acid and carnosol being not detectable in the basolateral medium after 2 and 4 h. However, when the basolateral fraction, collected after 8 h was added to the activated macrophages, a significant reduction in all measured cytokines was found, and even more strongly for IL-6. Furthermore, the incubation of activated macrophages with the absorbed fraction of rosemary extract after 12 h, reduced the secretion of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10 very considerably, 50% or more. The results indicated that inhibition of the secretion of measured cytokines by 8 and 12 h basolateral fractions of rosemary extract, correlated to an increase in carnosic acid in the fractions. Also, to a lesser extent, it correlated to the small amount of carnosol detected after 12 h in the basolateral samples.

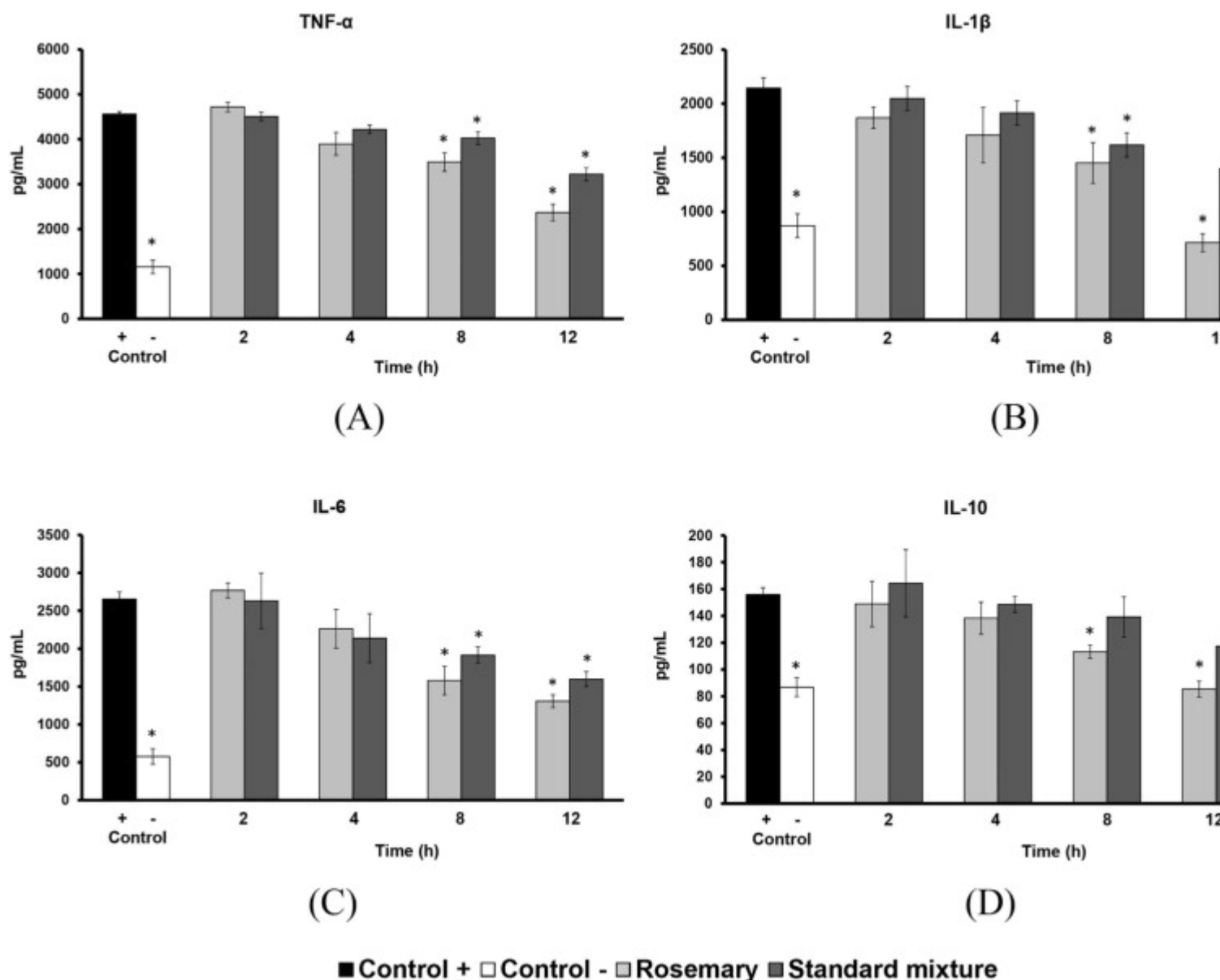


Fig. 3. Concentration of TNF- $\alpha$  (A), IL-1 $\beta$  (B), IL-6 (C) and IL-10 (D) secreted by THP-1/M activated with LPS in presence of the bioavailable fractions of supercritical rosemary extract or standard mixture. Each bar is the mean of three determinations  $\pm$  standard deviation. \*Denotes statistical differences between Control+LPS and the other samples at  $p < 0.05$ .

In order to determine if the inhibition in the secretion of measured cytokines was only related to the presence of carnosic acid and carnosol in the basolateral fractions, the anti-inflammatory activity of the basolateral fraction of the mixture of carnosic acid and carnosol was also determined under the same conditions. The data also showed that only fractions obtained after 8 and 12 h decreased the secretion levels of all the cytokines tested, although in a lesser extent than when rosemary extract was employed.

These results demonstrated that the basolateral fraction from rosemary supercritical extract and the mixture of carnosic acid and carnosol presented an important anti-inflammatory activity, since both reduced the secretion of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6. However, the anti-inflammatory activity of the basolateral fraction of rosemary extract was higher than the fraction from the mixture of carnosic acid and carnosol, indicating that in the rosemary extract other compounds with anti-inflammatory activity were presented either derived from the exposed sample or due to compounds secreted by Caco-2 as a response to the challenge. Earlier work developed from our research group described the presence of compounds as 1,8-cineole or camphor in the supercritical rosemary extract (Fornari, Vicente, Vázquez,



García-Risco, & Reglero, 2012), for which anti-inflammatory activity has been reported previously (Ehrnhöfer-Ressler et al., 2013; Santos & Rao, 2000). Moreover, Atul Bhattaram, Graefe, Kohlert, Veit, and Derendorf (2002) and Boyle, McLean, Brandon, and Wiggins (2005) reported the bioavailability of 1,8-cineole.

In conclusion, the results demonstrated that carnosic acid and carnosol included in a supercritical rosemary extract showed the same behavior as a mixture of these compounds. Furthermore, the basolateral fraction of this supercritical extract showed a significant anti-inflammatory activity. These results suggest a possible application of supercritical rosemary extracts, besides flavorants, in formulations for the prevention of inflammatory diseases.

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